ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2009, p. 1528–1531 Vol. 53, No. 4
0066-4804/09/$08.00+0 doi:10.1128/AAC.00971-08

Use of a Surface Plasmon Resonance Method To Investigate Antibiotic and Plasma Protein Interactions▽

Françoise Banères-Roquet,[1,2,3] Maxime Gualtieri,[1,2,3] Philippe Villain-Guillot,[1,2,3] Martine Pugnière,[1,2,3] and Jean-Paul Leonetti[1,2,3]*

Université Montpellier 1, Centre d'Études d'Agents Pathogènes et Biotechnologies pour la Santé (CPBS), Montpellier, France[1]; CNRS, UMR 5236, CPBS, 4 Bd Henri IV, CS 69033, F-34965 Montpellier, France[2]; and Université Montpellier 2, CPBS, F-34095 Montpellier, France[3]

Received 22 July 2008/Returned for modification 16 December 2008/Accepted 31 December 2008

The pharmacologic effect of an antibiotic is directly related to its unbound concentration at the site of infection. Most commercial antibiotics have been selected in part for their low propensity to interact with serum proteins. These nonspecific interactions are classically evaluated by measuring the MIC in the presence of serum. As higher-throughput technologies tend to lose information, surface plasmon resonance (SPR) is emerging as an informative medium-throughput technology for hit validation. Here we show that SPR is a useful automatic tool for quantification of the interaction of model antibiotics with serum proteins and that it delivers precise real-time kinetic data on this critical parameter.

Serum proteins play an important role in binding to many drugs, including antibiotics. In general, serum proteins decrease the free fraction of antibiotic available for the elimination of bacteria, since only the non-protein-bound molecules are pharmacologically active. The proteins involved in this sequestration are mainly human serum albumin (HSA), the most abundant serum protein (4% wt/vol); α-1-acid glycoprotein (AGP); and gamma globulin (5, 7, 11).

Currently, most of the reports of the inhibitory effects of serum proteins on antibiotics are derived from in vitro studies that have employed the MIC method (3, 9) or time-killing curves (16). The findings described in those reports correlate well with in vivo data (13) and are useful for evaluation of the potential of a new drug candidate. However, it is also necessary to rapidly and precisely characterize how a molecule binds to serum proteins in terms of affinity constants to drive the synthesis of new and more efficient analogs. A variety of physical techniques for measurement of the levels of protein binding have been proposed. The most classical are ultracentrifugation (3) and dialysis (3, 8); but other alternative techniques have been used, like circular dichroism analysis (1) and extrinsic fluorescence analysis (15). More recently, surface plasmon resonance (SPR) was proposed as a medium- to high-throughput alternative for evaluation of the kinetics of relatively lipophilic drugs that bind to human serum proteins in real time (14).

Antibiotics are characterized as having a relatively high hydrophilicity compared to the hydrophilicities of other drug classes. Consequently, antibiotics have lower affinities for serum proteins. Because the development of fast analytical methods that allow the measurement of antibiotic-serum protein interaction kinetics with a small amount of sample is desirable, we have evaluated if SPR can measure low affinities and how SPR can be used to prescreen rapidly libraries of antibiotic candidates for their propensity to bind to serum proteins.

MATERIALS AND METHODS

Bacterial strains, antimicrobial agents, and media. *Staphylococcus aureus* reference strain CIP 76.25 (ATCC 25923) was used. *S. aureus* was grown, subcultured, and quantified in Mueller-Hinton broth and on Mueller-Hinton agar (Difco Laboratories, Detroit, MI). Antimicrobial agents belonging to different antibiotic classes were selected. The antibiotics rifampin (rifampicin), vancomycin, minocycline, fusidic acid, novobiocin, lincomycin, ofloxacin, cefotaxime, and erythromycin and the nonantibiotic molecule warfarin (used as a control) were purchased from Sigma-Aldrich (St. Louis, MO). Depending on the experiment, the Mueller-Hinton broth medium was supplemented with HSA, AGP from human plasma, or gamma globulin from human blood (Sigma-Aldrich).

SPR experiments. SPR experiments were performed at 25°C with a BIACORE 3000 apparatus (GE Healthcare, Biacore AB, Uppsala, Sweden).

Procedure for protein immobilization. HSA diluted to 40 μg/ml in 10 mM acetate buffer, pH 5.2, was immobilized on CM5 sensor chips by the use of amine-coupling chemistry. The surface was blocked with 1 M ethanolamine, pH 8.0, and was washed with three 30-s pulses of 50 mM NaOH to remove free HSA. Human gamma immunoglobulin (gamma globulins; Sigma-Aldrich) was immobilized on flow cell 3 by the same method. 2-(2-Pyridinyldithio)ethaneamine hydrochloride (PDEA)-modified AGP was immobilized on flow cell 4 by a standard surface thiol-coupling procedure (10). The immobilization levels ranged from 9,000 to 12,000 resonance units (RU) for gamma globulins and HSA and from 7,000 to 10,000 RU for AGP.

Ranking experiments. Drugs (rifampin, vancomycin, minocycline, fusidic acid, novobiocin, lincomycin, ofloxacin, cefotaxime, erythromycin, and warfarin) were prepared as 10 mM stock solutions in 100% dimethyl sulfoxide (DMSO). They were then diluted in phosphate-buffered saline (PBS) or PBS containing DMSO to reach final concentrations of 250 μM in 5% DMSO. Binding studies were conducted in PBS containing 5% DMSO and at a flow rate of 50 μl/min. For the ranking of drug compound binding to HSA, gamma immunoglobulin, and AGP, randomized duplicate samples of drugs at 250 μM were injected for 30 s over the immobilized proteins or a reference surface without protein. The surfaces were then washed with the running buffer until complete regeneration was achieved. To clean the flow system, a bypass wash was performed with 50% DMSO and 5% DMSO between each injection. Buffer blanks were injected before each drug injection, and the binding responses were corrected for DMSO bulk differences by the use of calibration curves (eight DMSO solutions with between 4.5 and 5.8% DMSO) and were normalized to the same 10,000-RU immobilization level for HSA, AGP, and gamma globulins.

Kinetic experiments. The compounds were injected at various concentrations (from 400 μM to 0.1 μM in twofold dilutions) over the reference and HSA flow

* Corresponding author. Mailing address: CNRS-CPBS/UMR 5236, Faculté de Pharamcie, 15 Av Charles Flahault, 34 195 Montpellier Cedex 5, France. Phone: 33 4 67 61 37 46. Fax: 33 4 67 61 37 87. E-mail: Jean-Paul.Leonetti@univ-montpl.fr.

▽ Published ahead of print on 21 January 2009.

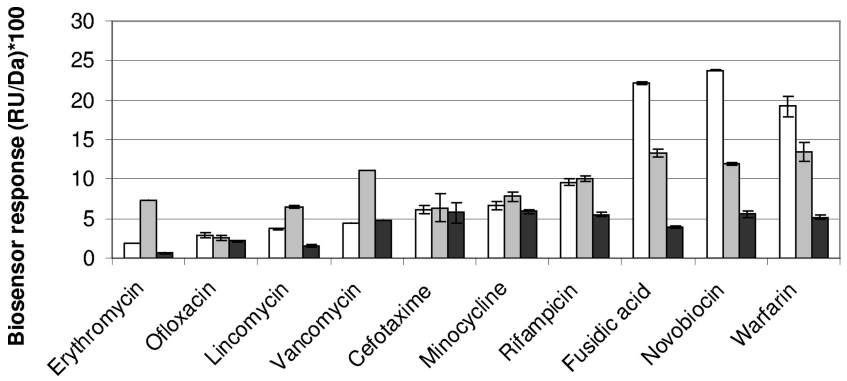

FIG. 1. Ranking of the interactions of reference antibiotics on HSA, AGP, and gamma globulins. The binding of rifampin, vancomycin, minocycline, fusidic acid, novobiocin, lincomycin, ofloxacin, cefotaxime, and erythromycin to HSA, AGP, and gamma globulins was tested by SPR. The nonantibiotic molecule warfarin was included as a control in all the experiments. Data were normalized to the same 10,000-RU immobilization level for HSA (white bars), AGP (gray bars), and gamma globulins (black bars). The experiments were done in triplicate, and the results are presented as the mean value ± standard error (the error was low, and the bars showing the error are not always visible).

cells for 30 s at a flow rate of 50 μl/min. Each cycle consisted of the injection of a blank buffer before the injection of the antibiotic (30 s of association, 30 s of dissociation), and the system was cleaned by using a bypass wash procedure with 50% DMSO and 5% DMSO. The data (collected at a rate of 2.5 Hz) obtained for the reference flow cell was subtracted from that obtained for the HSA flow cell. The responses from the injections of drug compounds were extracted 10 s after the beginning of the injection. These responses were further corrected for the effects of DMSO by use of the calibration curves, and the final response values were used to plot the dose-response and for K_D (equilibrium dissociation constant) determinations, as described by Frostell-Karlsson et al. (6). The level of binding of warfarin at 50 μM was measured at different moments before, during, and after each drug sample to check the response stability of the protein-coated surface.

Fraction of sites occupied by antibiotics. The dose-response curves were obtained by plotting the RU responses (corrected from the DMSO bulk response) against the drug concentrations or by plotting the fraction of the sites occupied against the drug concentrations.

The fraction of the sites occupied by the antibiotics on HSA was calculated as follows: $(\text{Req} \cdot \text{MW}_{\text{HSA}})/(\text{Rim} \cdot \text{MW}_{\text{drug}})$, where Req is the response value (in RU; corrected from the DMSO bulk response), Rim is the HSA immobilization level (in RU), and MW is molecular weight.

In vitro susceptibility studies. The MICs of rifampin, vancomycin, minocycline, fusidic acid, novobiocin, lincomycin, ofloxacin, cefotaxime, and erythromycin for *S. aureus* CIP 76.25 were determined in duplicate by standard microdilution methods of the Clinical and Laboratory Standards Institute (formerly NCCLS) (12).

Protein binding. The impact of serum protein binding was assessed by examining the impacts of human serum, HSA, AGP, and gamma globulin on the activities of nine antibiotics in vitro. To determine the effects of serum protein on in vitro antimicrobial properties, MIC tests were performed in the presence of 50% human serum, 4% HSA, 0.1% AGP, or 1.6% gamma globulins. The concentrations of the serum proteins used for the in vitro studies were selected on the basis of the normal physiological concentrations. A reduced potency (higher MIC) in the presence of serum or protein serum was presumed to be caused by drug binding to serum proteins. The ratio of the MICs measured in the presence of plasma protein to the standard MIC was used to estimate the effects of the plasma proteins.

RESULTS

Ranking of antibiotic-serum protein interactions. Nine antibiotics with different physicochemical properties were used to set up an experimental protocol to determine the HSA, AGP, and gamma globulin binding levels. Some of these have previously been characterized by microbiological methods, but in most the cases, the binding kinetics and affinities are unknown. HSA, AGP, and gamma globulins differ in their molecular weights and their capacities to coat the surface of the sensor chip, which impairs the direct comparison of the binding responses. An average coating of 9,000 to 12,000 RU was observed, and we have normalized the response to an average level of 10,000 RU. Equilibrium responses were collected in randomized duplicate for the nine antibiotics at 250 μM. The association and dissociation were achieved rapidly, and no regeneration of the surface was necessary. At that concentration, all of the compounds gave measurable responses. We show that novobiocin and fusidic acid gave higher responses on HSA than on AGP (Fig. 1). Vancomycin, erythromycin, and lincomycin, to a lesser extent, gave higher levels of binding to AGP than to HSA. Cefotaxime, minocycline, and rifampin bound to HSA and AGP at quite similar levels. The nonantibiotic molecule warfarin was used as a control throughout the experiments. The results of the ranking experiments with gamma globulins were less conclusive, since all the drugs tested exhibited low-level binding responses (<5) at the maximum coating level.

Experiments were performed with the antimicrobials to compare the SPR ranking with the inhibitory effects of serum, HSA, AGP, and gamma globulin. HSA, AGP, and gamma globulins were used at concentrations equivalent to their respective concentrations in serum, although these purified proteins are unlikely to give responses strictly comparable to the ones expected in a more complex environment. The strongest HSA binders, novobiocin and fusidic acid, were the most affected by the presence of HSA in the culture broth; a 256-fold increase in the MICs was observed in the presence of 4% HSA, and a 1,000-fold increase was observed in the presence of serum (Table 1). Cefotaxime and minocycline demonstrated almost identical data for the biosensor response in the presence of the three proteins, and the effects of the proteins on the activities of the antimicrobials against *Staphylococcus aureus* were comparable. However, their values for percent binding to serum taken from the literature rank them as strong binders. This discrepancy can potentially be explained by differences in the techniques used to obtain the measurements (i.e., ultracentrifugation and dialysis) or the origin of the serum.

TABLE 1. Effects of serum proteins on different reference antibiotics

Antibiotic	Standard MIC (μg/ml)	MIC (μg/ml) with the following plasma protein:			
		50% human serum	4% HSA	0.1% AGP	1.6% gamma globulin
Ofloxacin	0.195	2	1	1	2
Cefotaxime	0.781	4	1	1	2
Lincomycin	0.391	2	2	2	1
Erythromycin	0.195	2	2	2	2
Minocycline	0.195	4	4	1	1
Rifampin	0.049	4	4	1	2
Vancomycin	0.781	2	4	2	1
Novobiocin	0.049	1,024	256	2	2
Fusidic acid	0.049	1,024	256	2	2

The strongest AGP binders (novobiocin, fusidic acid, vancomycin, lincomycin, and erythromycin) were also the most affected by the presence of AGP, with the increase in the MICs being twofold. The effects of gamma globulins on the MICs (Table 1) are almost uniform according to the SPR data and are limited to a twofold increase in the MIC ratio.

Kinetics and fraction of sites occupied. SPR is well suited to determination of how many binding sites are involved in the interactions between drugs and serum proteins. HSA is known to have two main sites of drug binding, and warfarin is a well-characterized HSA ligand that preferentially binds to site I (4). Frostell-Karlsson and colleagues (6) and Rich et al. (14) used warfarin as a model molecule to validate an SPR method for drug-HSA binding constant determination from dose-response measurements. We expanded the same procedure for antibiotic molecules to determine the fraction of sites occupied on HSA and AGP.

The ranges of drug concentrations that allowed the occupancy of 0.5 to 2 sites per protein molecule are listed in Table 2 for the nine antibiotics. Novobiocin was a strong HSA binder, occupying up to 2 sites at 180 μg/ml. Fusidic acid and rifampin occupied 1.5 sites at 215 and 320 μg/ml, respectively. Since most of the drugs occupied more than one binding site on HSA, they generated rather complex binding kinetics over a concentration range from 0.1 μg/ml to 250 μg/ml. This prevented us from evaluating the affinities for all the sites. We have estimated the K_D only for the first site from the binding curve. They ranged from 12 to 150 μM. On AGP, the fraction of sites occupied was less than 1 for all the drugs (data not shown).

The binding profiles of the different drugs are informative about their properties, and the profiles for three of them are shown in Fig. 2 as illustrative examples. Rifampin gave a linear dose-response binding curve over a wide range of concentrations (1 to 300 μg/ml) and did not saturate HSA (not more then 20 RU of binding at 300 μg/ml). Novobiocin and fusidic acid, the strongest HSA binders in the SPR ranking experiment, also showed nonsaturating binding profiles at between 0.1 and 300 μg/ml, but we have observed two- and fourfold more binding at 300 μg/ml, respectively.

DISCUSSION

The technique presented here was successfully used to evaluate the propensity of binding of reference antibiotics to HSA, AGP, and gamma globulin. Serum proteins control the availability and the distribution of most drugs. HSA principally interacts with acidic molecules (7) like fusidic acid and novobiocin, while AGP is a major target for the binding of a wide variety of basic and neutral drugs (17). By binding to HSA, many hydrophobic drugs with low solubilities can be transported to reach their target tissues effectively. A decrease in the concentration of molecules readily available to interact with the true target protein results in a decrease in the antimicrobial activity of the molecule. This is why the evaluation of the binding of antibiotics to serum proteins is a critical aspect in the development of this category of drugs.

In our assay, the relatively hydrophilic antibiotics tested had lower affinities for serum proteins then most of the other classes of drugs tested by SPR (6, 14). However, we show that the technique is robust enough to be used to test this drug family. The antibiotics with the strongest ability to physically interact with serum proteins are the most affected by the presence of serum in MIC assays (2, 3; unpublished data). As expected, HSA was the most abundant plasma protein, accounted for approximately 60% of the total proteins, and was the major contributor to the binding of these reference antibiotics. The binding of drugs to multiple sites on HSA is well documented in the literature (7). This correlates with the complex binding kinetics observed in our experiments and prevented us from evaluating the affinities for all the sites. However, the occupancy of the drug binding sites by an antibiotic at

TABLE 2. Fraction of sites occupied on HSA

Drug	Fraction of sites occupied on HSA[a] for a range of drug concn (μg/ml)	C_{max} (μg/ml)[b]	% Binding to serum[b]
Ofloxacin	0.5 (18–36),[c] 1 (>145)	2.6	25
Cefotaxime	0.5 (22–44), 1 (~180)	4.9–17	3
Lincomycin	0.5 (130–180)	1.8–2.5	75
Erythromycin	0.5 (146–220)	3	19–25
Minocycline	0.5 (~50), 1(135–180)	2.6	54–80
Rifampin	0.5 (80–120), 1 (~245), 1.5 (>325)	11	64
Vancomycin	0.5 (~285), 1 (430–570)	30	10–19
Novobiocin	0.5 (7–15), 1 (~60), 1.5 (~120), 2 (180–250)	62.5	
Fusidic acid	0.5 (~20), 1 (~160), 1.5 (215–430)	52	97–98

[a] The fraction of sites occupied on HSA was calculated as described in the text.
[b] C_{max} and percent binding to serum are compilations of data obtained for different animal species (2, 3).
[c] The drug concentration ranges are given in parentheses.

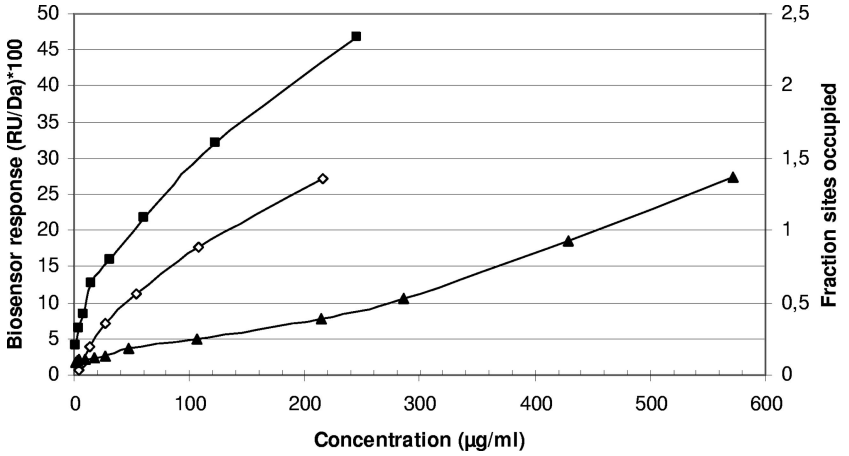

FIG. 2. Dose-response binding of rifampin, fusidic acid, and novobiocin on HSA. The concentration-dependent binding of rifampin (▲), fusidic acid (◇), and novobiocin (■) to HSA was monitored by SPR. The binding response is presented on the left axis, and the fraction of the sites occupied on the protein is shown on the right axis.

a given concentration can readily be compared to the maximum concentration of the antibiotic in serum (C_{max}). In the case of erythromycin, a concentration of about 150 μg/ml was necessary to occupy 50% of the HSA sites. However, the C_{max} of this antibiotic does not exceed a few μg/ml (2). We conclude that the binding occurs over a concentration range far higher than the concentration reached in serum. Alternatively, one molecule of novobiocin binds to one molecule of HSA at a concentration of 60 μg/ml, and its C_{max} is in same concentration range (2), which makes the observation directly relevant for the pharmacologist.

In conclusion, SPR is not a substitute for classical techniques since the binding of a drug to a single purified protein like HSA does not always mimic the behavior of the same drug in serum. However, HSA is by far the major contributor to these interactions, and SPR efficiently discriminates between antibiotics that are weak and strong binders to HSA. These characteristics could help with the rapid and automatic prescreening of larger libraries of new antibiotic candidates.

ACKNOWLEDGMENTS

This work was supported by the DGA and the CNRS. Villain-Guillot was supported by a postgraduate fellowship from the DGA.

REFERENCES

1. **Ascoli, G., C. Bertucci, and P. Salvadori.** 1995. Stereospecific and competitive binding of drugs to human serum albumin: a difference circular dichroism approach. J. Pharm. Sci. **84:**737–741.
2. **Bryskier, A.** 1999. Antibiotics, antibacterial agents, and antifungals, vol. 1. American Society for Microbiology, Washington, DC.
3. **Craig, W. A., and B. Suh.** 1996. Protein binding and the antimicrobial effects: methods for the determination of protein binding. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore, MD.
4. **Fehske, K. J., U. Schläfer, U. Wollert, and W. E. Müller.** 1982. Characterization of an important drug binding area on human serum albumin including the high-affinity binding sites of warfarin and azapropazone Mol. Pharmacol. **21:**387–393.
5. **Fitos, I., J. Visy, F. Zsila, G. Mády, and M. Simonyi.** 2007. Conformation selectivity in the binding of diazepam and analogues to alpha1-acid glycoprotein. Bioorg. Med. Chem. **15:**4857–4862.
6. **Frostell-Karlsson, A., A. Remaeus, H. Roos, K. Andersson, P. Borg, M. Hamalainen, and R. Karlsson.** 2000. Biosensor analysis of the interaction between immobilized human serum albumin and drug compounds for prediction of human serum albumin binding levels. J. Med. Chem. **43:**1986–1992.
7. **Ghuman, J., P. A. Zunszain, I. Petitpas, A. A. Bhattacharya, M. Otagiri, and S. Curry.** 2005. Structural basis of the drug-binding specificity of human serum albumin. J. Mol. Biol. **353:**38–52.
8. **Huang, J.** 1983. Errors in estimating the unbound fraction of drugs due to the volume shift in equilibrium dialysis. J. Pharm. Sci. **12:**1368–1369.
9. **Laue, H., T. Valensise, A. Seguin, S. Hawser, S. Lociuro, and K. Islam.** 2007. Effect of human plasma on the antimicrobial activity of iclaprim in vitro. J. Antimicrob. Chemother. **60:**1388–1390.
10. **Löfås, S., B. Johnsson, Å. Edström, A. Hansson, G. Lindquist, R. Müller Hillgren, and L. Stigh.** 1995. Methods for site controlled coupling to carboxymethyldextran surfaces in surface plasmon resonance sensors. Biosens. Bioelectron. **10:**813–822.
11. **McElnay, J., and P. D'Arcy.** 1983. Protein binding displacement interactions and their clinical importance. Drugs **25:**495–513.
12. **National Committee for Clinical Laboratory Standards.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 6th ed. NCCLS document M7-A6. National Committee for Clinical Laboratory Standards, Wayne, PA.
13. **Peterson, L. R., J. A. Moody, C. E. Fasching, and D. N. Gerding.** 1989. Influence of protein binding on therapeutic efficacy of cefoperazone. Antimicrob. Agents Chemother. **33:**566–568.
14. **Rich, R. L., Y. S. N. Day, T. A. Morton, and D. G. Myszka.** 2001. High-resolution and high-throughput protocols for measuring drug/human serum albumin interactions using BIACORE. Anal. Biochem. **296:**197–207.
15. **Sudlow, G., D. J. Birkett, and D. N. Wade.** 1973. Spectroscopic techniques in the study of protein binding: the use of 1-anilino-8-naphthalenesulphonate as a fluorescent probe for the study of the binding of lophenoxic and lopanoic acids to human serum albumin. Mol. Pharmacol. **9:**649–657.
16. **Zeitlinger, M. A., R. Sauermann, F. Traunmuller, A. Georgopoulos, M. Muller, and C. Joukhadar.** 2004. Impact of plasma protein binding on antimicrobial activity using time-killing curves. J. Antimicrob. Chemother. **54:**876–880.
17. **Zsila, F., and Y. Iwao.** 2007. The drug binding site of human alpha1-acid glycoprotein: Insight from induced circular dichroism and electronic absorption spectra. Biochim. Biophys. Acta **1770:**797–809.